(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
10 May 2001 (10.05.2001)

PCT

(10) International Publication Number
WO 01/32139 A2

(51) International Patent Classification[7]: A61K 9/00

(21) International Application Number: PCT/EP00/11236

(22) International Filing Date:
6 November 2000 (06.11.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
99203673.1 5 November 1999 (05.11.1999) EP

(71) Applicant *(for all designated States except US)*: **SEED CAPITAL INVESTMENT-2 (SCI-2) B.V.** [NL/NL]; Prof. Bronkhorstlaan 25, NL-3723 MB Bilthoven (NL).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **BURGER, Koert, Nico, Jan** [NL/NL]; Edelhertakker 28, NL-3994 ER Houten (NL). **STAFFHORST, Rutger, Willibrordes, Henricus, Maria** [NL/NL]; Bergstraat 88, NL-5051 HD Goirle (NL). **DE KRUIJFF, Ben** [NL/NL]; Lange Uitweg 40, NL-3999 WL Tull En 'T Waal (NL).

(74) Agent: **VAN SOMEREN, Petronella, Francisca, Hendrika, Maria**; Arnold & Siedsma, Sweelinckplein 1, NL-2517 GK The Hague (NL).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

**Published:**
— *Without international search report and to be republished upon receipt of that report.*

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

WO 01/32139 A2

(54) Title: METHOD FOR THE FORMULATION OF SUBSTANCES HAVING A LOW WATER-SOLUBILITY AND LIPOPHILICITY AND FORMULATION THUS OBTAINED

(57) Abstract: The present invention relates to a method for encapsulating substances, in particular drugs, that have a low water-solubility and lipophilicity, which method comprises providing a lipid system comprising one or more negatively charged lipid(s); combining the lipid system with the substance(s) in a medium at a low total solutes concentration; subjecting the mixture thus obtained to one or more cycles of freezing and thawing to produce lipid-coated aggregates of the substance(s), and optionally removing the free (non-enclosed) substance(s). The method is in particular suitable for encapsulating cisplatin.

# METHOD FOR THE FORMULATION OF SUBSTANCES HAVING A LOW WATER-SOLUBILITY AND LIPOPHILICITY AND FORMULATION THUS OBTAINED

The present invention relates to a new method for the formulation of substances having a low water-solubility and lipophilicity in general and cisplatin in particular. The invention further relates to the formulation thus obtained.

cis-Diamminedichloroplatinum(II) (cisplatin) is one of the most widely used agents in the treatment of solid tumors, and particularly effective against testicular and ovarian cancers. However, expansion of the clinical utility of cisplatin has been limited by its toxicity, as well as by the occurrence of intrinsic and acquired resistance in many common tumor types. the toxic side effects associated with the clinical use of cisplatin include nausea and vomiting, nephrotoxicity, ototoxicity, neuropathy and myelosuppression.

In addition, cisplatin is a highly reactive molecule that interacts with a variety of extracellular as well as intracellular biomolecules. In particular sulphur- and nitrogen-donor atoms have a high affinity for cisplatin. As a result, cisplatin binds extensively to plasma and tissue proteins which leads to a fast reduction in bioavailability and in the effective inactivation of a large part of the administered dose. In vitro studies indicate that 96% of cisplatin is bound to plasma proteins within 24 hrs. The remaining non-complexed or free cisplatin enters the cell by a combination of passive diffusion and protein-mediated uptake, and then interacts with intracellular substrates: thiols such as glutathione, methionine-containing proteins and peptides, and with the guanine bases in RNA and DNA. Only a few percent of the cisplatin entering the cell is able to bind to DNA.

Thus, the clinical use of cisplatin and many of its analogs faces three major problems, (i) serious dose-

limiting toxicities in particular nephrotoxicity and neurotoxicity, (ii) rapid inactivation of the drug as a result of complexation to plasma and tissue proteins, and (iii) the frequent occurrence of platinum resistance.

In general, these problems can be reduced by shielding of a drug from the extracellular environment by means of a lipid coating, such as the so-called liposomes. Liposomes are micro-particulate or colloidal carriers, typically 0.05-5.0μm in size which form spontaneously when certain lipids are hydrated in aqueous media. Liposomes are composed of relatively biocompatible (non-toxic) and biodegradable material, and consist of an aqueous volume entrapped by one or more bilayers of natural and/or synthetic lipids. Depending on their lipophilicity, drugs can be encapsulated in the phospholipid bilayer, in the aqueous compartment or at the bilayer interface.

After intravenous, interstitial or intracavitary administration, the liposomes in general interact with cells of the target organ resulting in the local release of the encapsulated drug. The drug is either released extracellularly, after destabilisation of the liposomal carrier at the cell surface, or intracellularly, after endocytic uptake of the carrier by the target cell.

Liposomes have been used as carriers of numerous pharmacologically active agents, such as antineoplastic and antimicrobial drugs, steroids, vaccines and genetic material.

However, in many cases this approach fails because of inefficient encapsulation of the drug in lipid formulations resulting in low drug uptake by the tumor. This is particularly true for lipid formulations of cisplatin: the low water-solubility and lipophilicity of cisplatin result in lipid formulations with a very low drug-to-lipid ratio.

It is therefore the object of the present invention to provide a method allowing an extremely

efficient encapsulation of substances, in particular drugs, that are poorly water-soluble and have a low lipophilicity in general and cisplatin in particular in a lipid formulation.

In the research that led to the present invention it was found that a method that is based on one or more freezing and thawing cycles of a concentrated solution of cisplatin in the presence of negatively charged phospholipids generates small aggregates ("nanoparticles") of cisplatin covered by a single (and occasionally two or three) lipid bilayer(s). The lipid-coated nanoparticles of cisplatin have an unprecedented drug-to-lipid ratio and an in vitro cytotoxicity up to 1000-fold higher than the free drug. The encapsulation efficiency of 1-4 mg cisplatin per μmol lipid is extremely high.

More in particular, these results were obtained by hydration of a dry lipid film composed of equimolar amounts of dioleoyl-phosphatidylserine (PS) and dioleoyl-phosphatidylcholine (PC), with a buffered solution (pH 7.4) of 5 mM cisplatin followed by 10 freeze-thaw (FT) cycles using ethanol/dry-ice (-70°C) and a waterbath (37°C). Subsequently, the free (extravesicular) cisplatin was removed by repeated centrifugation and resuspension of the membrane pellet in a buffer. The resulting cisplatin-containing lipid suspension (cisPt-PS/-PC) was extremely cytotoxic with an $IC_{50}$ of ~2 nM as compared to 0.5 μM for the free drug (conventional cisplatin). A lipid suspension not loaded with cisplatin (blank) was not cytotoxic, and mixing conventional cisplatin with the blank lipid suspension did not result in an increase in cytotoxicity of cisplatin.

To determine the reason for the extreme cytotoxicity of the lipid formulation of cisplatin, the effects of a number of variations in the standard protocol, on the in vitro anti-tumor activity and on the encapsulation efficiency were studied. Omitting the freeze-thaw step or leaving out the negatively charged PS

in the lipid mixture, resulted in a dramatic decrease in the cytotoxicity of the formulation. This dramatic decrease in cytotoxicity was paralleled by a similar decrease in the encapsulation efficiency or drug-to-lipid ratio suggesting a direct relation between drug-to-lipid ratio and cytotoxicity: omitting freeze-thawing or leaving out PS typically decreased the drug-to-lipid mole ratio 10-fold and 4-fold, respectively.

Using as a standard protocol, an equimolar mixture of PS and PC, and a buffered solution of 5 mM cisplatin (10 mM Pipes-NaOH, 1 mM EGTA, pH 7.4), the cisplatin-to-lipid mol ratio of the lipid formulation was ~0.5, which translates into a theoretical intravesicular concentration of cisplatin in excess of 30 mM (calculation based on an encapsulated volume of 15 liter/mol phospholipid). Thus, the intravesicular concentration by far exceeded the solubility limit of cisplatin (~8 mM in chloride-free media), and strongly suggested that the lipid formulations contained aggregates of cisplatin. Therefore, the formulations were examined with cryo-electron microscopy and indeed small aggregates, so-called "nanoparticles", of cisplatin coated by a thin lipid layer were observed.

Even higher drug-to-lipid ratios of ~2.5 could be obtained by preparing the lipid formulation using a solution of 5 mM cisplatin in $H_2O$ instead of buffer, and these preparations were used to isolate the lipid-coated nanoparticles by pelleting through high density sucrose cushions. Electron microscopical analysis confirmed that the pellet existed of lipid-coated nanoparticles, and that lipid vesicles were virtually absent. The majority of the particles had an elongated shape measuring about 46 nm by 86 nm (lipid coat inclusive), and the drug-to-lipid mol ratio of the pellet fraction was exceptionaaly high, ~11, which translates into ~3.3 mg cisplatin per $\mu$mol phospholipid.

Analysis of the mechanism underlying the formation of these so-called "nanopills" indicates that

the method may be generalized to other substances, such as drugs, showing low water-solubility and lipophilicity.

The present invention therefore relates to a method for encapsulating substances, in particular drugs, that have a low water-solubility and lipophilicity, which method comprises:

a) providing a lipid system comprising one or more negatively charged lipid(s);

b) combining the lipid system with the substance(s) in a medium at a low total solutes concentration;

c) subjecting the mixture thus obtained to one or more cycles of freezing and thawing to produce lipid-coated aggregates of the substance(s); and

d) optionally removing the free (non-enclosed) substance(s).

The invention can be applied for single substances or for mixtures of substances. To indicate this the term "substance(s)" is used.

The medium having a low total solutes concentration is preferably a medium having a maximum total solutes concentration of 0.1 molar. More preferably the total solutes concentration is not more than 0.02 molar.

Preferably, the substance(s) is(are) used in a high concentration. A high concentration is a concentration that does not lead to the formation of macromolecular aggregates of the substance(s) in the solution of the substance(s), i.e. before the freeze thaw cycle.

Preferably, the concentration lies close to the solubility limit of the substance(s), wherein "close" means either closely below or closely above the solubility limit. However, concentrations well above the solubility limit may under certain conditions still be suitable, provided that no macromolecular aggregates exist in the starting solution of the substance. Very

small aggregates may however serve as nucleation sites for aggregate formation of the invention and are as such also allowed in the medium.

The solubility limit of a substance depends on various parameters, such as the medium used, the pH and the temperature. It is clear that the solubility limit intended here is the limit existing at the conditions used in the method of the invention.

There is no lower concentration limit for the solution of the substance(s). However, it is practical to choose a concentration at which the method of the invention can still be efficiently performed.

The lipid system can be a single or a mixed system. The latter system comprises at least 20 mol%, preferably 50 mol% negatively charged lipid(s). The lipid system can take the form of a film, but preformed liposomes are equally well suited.

Combining the lipid system with the drug can thus be effected by hydrating a dry film of the lipid system with a solution of the drug. Alternatively, the dry lipid film is first hydrated to obtain preformed liposomes after which the drug is added. Both methods lead to similar results.

According to the invention at least one cycle of freezing and thawing is necessary, but multiple cycles, preferably 5 or more typically 10 are also possible.

The method of the invention may further comprise the step of isolating the lipid-coated drug aggregates e.g. by high-speed centrifugation (pelleting) through sucrose cushions, or, alternatively, by low-speed centrifugation in the absence of sucrose cushions.

This method is in particular useful when the substance is the drug cisplatin. Other substances that can be encapsulated by the method of the invention are poorly water-soluble hydrophilic substances such as the magnetic resonance and X-ray imaging agents based on Europium or Gadolinium.

The lipids in the mixture are preferably phosphatidylserine (PS) and phosphatidylcholine (PC), carrying dioleoyl fatty acids. Alternatively, the acyl chains are other unsaturated or saturated fatty acids, or other aliphatic hydrocarbons. Alternative negatively charged lipids are phosphatidic acid (PA), phosphatidylglycerol (PG), phosphatidylinositol (PI), and other negatively charged amphiphiles. Alternative neutral lipids are phosphatidylethanolamine (PE), PE derivatized to polyethyleneglycol or to other hydrophilic polymers, sphingomyelin (SM), and cholesterol, and other net neutral amphiphiles.

The solution containing the substance to be encapsulated, in particular a drug, may be a buffered solution of an approximately neutral pH, in particular a pH of about 7.4. Alternatively, the substance may be dissolved in water.

In a further embodiment of the invention a helper substance may be present in the drug solution. The helper substance is preferably positively charged. Conditions of the method should be chosen at which positively charged species of the substance (or helper substance) are present.

The freeze-thaw cycles may be effected in manners known to the skilled person, but a practical mode comprises freezing in ethanol/dry ice and thawing in a waterbath of 37°C.

The dry lipid film can be provided according to methods known in the art, as for example described in D. Lichtenberg, and Y. Barenholz (1988) Liposomes: preparation, characterization, and preservation. Methods Biochem. Anal. 33, 337-462.

Removal of the extravesicular drug can suitably be effected by repeated centrifugation and resuspension of the membrane pellet in an appropriate medium such as the medium (buffer, water) in which the drug was dissolved.

Based on using cisplatin as the substance to be encapsulated, the nanoparticle formation of the present invention is explained by the following mechanism (**Fig. 5**): a nearly saturated solution of cisplatin in $H_2O$, in the absence of added chloride, contains a mixture of the neutral dichloride- and dihydroxo-species of cisplatin which have a low solubility in water, and positively charged aquo-species of cisplatin with a much higher solubility. During freezing, solutes are excluded from the expanding ice phase and cisplatin is progressively concentrated in the residual fluid. The solubility limit of the neutral species of cisplatin is exceeded first and small aggregates form, which are subsequently covered by positively charged aquo-species of cisplatin. The highly positively charged cisplatin aggregates interact with the negatively charged lipid vesicles, and membranes reorganize to cover the surface of the aggregates resulting in lipid-coated nanoparticles of cisplatin. Only those aggregates of cisplatin that are completely covered by lipid, do not redissolve upon thawing.

This model is in excellent agreement with the experimental results which indicate that aggregation of cisplatin depends on freezing, and lipid coating on electrostatic interactions between positively charged species of cisplatin and negatively charged lipids (Table 1).

First, nanoparticle formation requires the presence of both negatively charged lipids and cisplatin, and critically depends on freeze-thawing. The choice of anionic and zwitterionic lipids and the stage at which cisplatin is added are not critical, PS can be replaced by phosphatidic acid (PA), and the same results are obtained adding cisplatin to a dry lipid film or to preformed liposomes.

Second, high chloride concentrations and alkaline pH, conditions that inhibit the formation of positively charged "aquated" species of cisplatin, prevent nanoparticle formation. At a chloride

concentration of 50 mM, nanoparticle formation can be restored completely by adding 10 % of preformed diaquated cisplatin just before freeze-thawing. Finally, high concentrations of salts such as $NaNO_3$, which prevent phase separation of ice and solutes and thus aggregation of cisplatin during freezing, strongly inhibits nanoparticle formation.

The reason for the higher cytotoxicity of the lipid-coated nanoparticles of cisplatin as compared to that of the free drug is probably a reduced drug inactivation; the lipid coat prevents the reaction of cisplatin with substrates present in the extracellular environment and at the cell surface. The lipid coating is either destabilized upon interacting with the cell surface, or, after endocytic uptake by the tumour cell (**Fig. 5**). Lipolytic enzymes present in the endosomal system digest the lipid-coat and release cisplatin, which, after crossing the endosomal membrane, ultimately interacts with nuclear DNA triggering cell death.

The amount of cisplatin encapsulated in the formulations of the invention is 2-3 orders of magnitude higher than that of any lipid formulation of cisplatin mentioned in the literature to date (Newman, M. S., Colbern, G. T., Working, P. K., Engbers, C. & Amantea, M. A. (1999) Cancer Chemother. Pharmacol. **43**, 1-7; Sur, B., Ray, R. R., Sur, P. & Roy, D. K. (1983) Oncology **40**, 372-376; Steerenberg, P. A., Storm, G., de, G. G., Claessen, A., Bergers, J. J., Franken, M. A. M., van Hoesel, Q. G. C. M., Wubs, K. L. & de Jong, W. H. (1988) Cancer Chemother. Pharmacol. **21**, 299-307). Moreover, the lipid-coated nanoparticles have an extremely high anti-tumor activity in vitro, up to 1000-fold higher than that of the free drug.

Lipid composition, surface charge, and size distribution of the lipid-coated nanoparticles can be manipulated and optimized for use in vivo as an anti-cancer formulation. A major problem of the lipid formulations of cisplatin used so far, appears to be the

low drug-to-lipid ratio, which limits the bioavailability of cisplatin in the tumor and which may result in low cytotoxicity and in regrowth of platinum resistant tumors. The method of the invention can overcome these problems.

Thus, two major goals in improving cancer treatment with cisplatin may be achieved, reduction of systemic toxicities and, in combination with dose-escalation, prevention and counteraction of platinum resistance.

The mechanism underlying the efficient encapsulation of cisplatin in lipid-coated nanoparticles strongly indicates that the same methodology may also prove successful in the encapsulation of other drugs which, like cisplatin, are difficult to enclose in liposomes because of their low water-solubility and low lipophilicity.

A particularly promising embodiment of the invention involves freeze-thawing a mixture of a poorly water-soluble neutral drug with a more water-soluble helper substance carrying a positive charge, in the presence of negatively charged phospholipids. During freezing, the solubility limit of the drug is exceeded first, and small aggregates form which are subsequently coated by the positively charged helper substance. Interaction of the positively charged aggregates with negatively charged lipids results in lipid-coated drug (-helper substance) aggregates which resist thawing.

Alternative embodiments of the method of the invention comprise the use of other amphipathic molecules instead of lipids and the induction of aggregates in other ways. These other ways of aggregation include the use of an oversaturated solution of the substance to be encapsulated and the addition of nucleation sites thereto. These two variants (other amphipathic molecules and other ways of aggregate induction) can be either used separately or be combined in the method as claimed.

Furthermore, it is possible according to the invention to manipulate the surface charge of the nanoparticles by making use of the coordination complex that forms between cisplatin and phosphatidylserine or cisplatin and phosphatidic acid as described in Speelmans et al. (Biochemistry, 36 (1997), 10545-10550).

The invention also relates to a formulation of a substance that is poorly water-soluble and has a low lipophilicity, obtainable by the method as claimed. In the formulation the substance takes the form of aggregates thereof coated with one or more bilayer(s) of lipids. Preferably, the majority of the aggregates are coated with one bilayer of lipids.

In a particular embodiment of the invention the formulation is a formulation of cisplatin, wherein the cisplatin takes the form of aggregates coated with one or more bilayer(s) of lipids. Preferably the majority of the aggregates are coated with one bilayer of lipids. The aggregates of cisplatin are preferably of an elongated shape measuring about 40-50, more in particular about 46 nm by 80 to 90, more in particular about 86 nm (lipid coat inclusive). Such cisplatin formulation is for example obtainable by the method of the invention.

The present invention will be further elucidated in the following example that is directed to the drug cisplatin. The invention is however more broadly applicable to other substances, such as drugs, that have a low water-solubility and low lipophilicity.

In the examples reference is made to the following figures:

**Fig. 1** Cytotoxicity towards human ovarian carcinoma cells.

(A) Lipid suspension of cisplatin (cisPt-PS/PC; ▲), conventional cisplatin (■), conventional cisplatin mixed with a blank (cisplatin-free) lipid suspension (□; same lipid concentration as in ▲), blank lipid suspension (dashed line).

(B) Variations on the standard protocol, omitting FT (▵), omitting PS (O). The cytotoxicity of conventional cisplatin (■) is not influenced by FT (□).

**Fig. 2** Morphology and subfractionation.

(A) Cryo-EM of cisPt-PS/PC reveals nanoparticles of cisplatin (marked by asterisk; MLV, multilamellar vesicle).

(B) Density gradient centrifugation of cisPt-PS/PC prepared in $H_2O$ (control prepared in the absence of cisplatin), high-density fraction (arrowhead), pellet (arrow). The pellet contained 17 ± 5 % (n=18) of the lipid loaded on the gradient.

(C) Cryo-EM, and

(D) negative stain EM of the pellet fraction (insert, at high electron dose). Micrographs at the same magnification, bar represent 100 nm.

**Fig. 3** Properties of the lipid-coat.

(A) $^{31}P$-NMR spectra of isolated nanoparticles (pellet) and control (PS/PC lipid suspension prepared in absence of cisplatin).

(B) Triton X100 addition (0.2 %) leads to immediate dissolution of nanoparticles.

**Fig. 4** Cytotoxicity and sizing of cisplatin nanoparticles.

(A) Cytotoxicity of conventional cisplatin (■), and of isolated nanoparticles before (▲) and after (▵) sizing.

(B) Dynamic light scattering analysis before (▲) and after (▵) sizing.

**Fig. 5** Model for nanoparticle formation and cell interaction. Partial hydrolysis of cisplatin in water yields positively charged weak acids: neutral and positively charged species of cisplatin are present in a suspension of negatively charged liposomes before FT (1), freezing results in ice-crystallization and aggregation of the neutral species (2), precipitation of positively charged species (3), interaction of positively charged cisplatin aggregates with negatively charged liposomes

(4), lipid-coated nanoparticles of cisplatin (5). Endocytic uptake of nanoparticles, destabilisation and intracellular release of cisplatin (6).

**EXAMPLE**

Encapsulation of cisplatin

**Materials and Methods**

**Lipid formulations of cisplatin**

Cisplatin (Sigma, St. Louis, MO) was dissolved in Pipes-EGTA buffer (10 mM Pipes-NaOH, 1 mM EGTA, pH 7.4) or MilliQ water and incubated in the dark overnight at 37°C to ensure full equilibration. Lipid dispersions (1.2 mM) were prepared by adding 5 mM cisplatin to a dry film of phospholipids (Avanti Polar Lipids, Inc., Birmingham, AL), incubating at 37°C for 15 minutes, followed by 10 freeze-thaw (FT) cycles using ethanol/dry-ice (-70°C) and a water bath (37°C).

Free (extravesicular) cisplatin was removed by repeated centrifugation (3-times, TLA 120.2, 40,000 rpm, 10 min, 20°C; Beckman Coulter, Inc., CA), resuspending the membrane pellet in Pipes-EGTA buffer.

Alternatively, a cisplatin-lipid dispersion in MilliQ water (1 ml) was loaded on top of a step gradient consisting of 1 ml each of 1.8 M, 0.6 M, and 0.2 M sucrose in Pipes-EGTA buffer, centrifuged (SW 60, Beckman Coulter, Inc., 55,000 rpm, 30 min, 4°C), and the pellet resuspended in MilliQ water. Filtered pellet fractions were obtained after high-pressure extrusion (Hope, M. J., Bally, M. B., Mayer, L. D., Janoff, A. S. & Cullis, P. R. (1986) Chem. Phys. Lipids **40**, 89-107) through polycarbonate filters (200 nm pore size), and reisolation of the pellet by sucrose gradient centrifugation.

**Encapsulation efficiency**

The phospholipid content was determined as described in (Rouser, G., Fleischer, S. & Yamamoto, A.

(1970) Lipids **5**, 494-496), and cisplatin was quantified by flameless atomic absorption spectroscopy, using a modifier solution of 0.5 % Triton X-100 in water, and $K_2PtCl_6$ (Sigma, St. Louis, MO) as a standard.

**Cytotoxicity assay**

Human-derived ovarian tumor cells IGROV-1 were grown on plastic in RPMI (Gibco, Glasgow, UK) supplemented with 25mM Hepes, 10% FCS, and 1% PenStrep. Cell lines were free of mycoplasma infections.

Formulations of cisplatin were diluted in RPMI without FCS to a cisplatin concentration of 233 μM. Tumor cell growth inhibition was determined using 96-wells plates and the sulforhodamine-B assay (Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., Warren, J. T., Bokesch, H., Kenney, S. & Boyd, M. R. (1990) J. Natl. Cancer Inst. **82**, 1107-1112). Approximately 1000 cells were seeded per well, the cisplatin formulations were added after 48 h (20 concentrations, in triplicate), and the cells further incubated for 120 h at 37°C.

Data were fitted to a sigmoidal dose-response curve (variable slope) using GraphPad Prism Software (San Diego, CA).

**Electron microscopy**

Cisplatin-lipid dispersions were visualized using bare- or lacey carbon grids, vitrification in melting ethane, and cryo-electron microscopy (cryo-EM) at low dose according to (Frederik, P. M., Stuart, M. C., Bomans, P. H. & Busing, W. M. (1989) J. Microsc. **153**, 81-92; Templeton, N. S., Lasic, D. D., Frederik, P. M., Strey, H. H., Roberts, D. D. & Pavlakis, G. N. (1997) Nat. Biotechnol. **15**, 647-652). Negative stain EM was performed using uranyl acetate following standard procedures.

**Miscellaneous procedures**

Diaquated cisplatin was prepared as described in (Appleton, T. G., Berry, R. D., Davis, C. A., Hall, J. R. & Kimlin, H. A. (1984) Inorg. Chem. **23**, 3514-3521) by incubating cisplatin with $AgNO_3$. Large unilamellar vesicles were prepared by extrusion according to (Hope et al. (1986), supra). Dynamic light scattering analysis of particle size was performed using a Zetasizer 3000 (Malvern Instruments Ltd., Malvern, UK). $^{31}P$-NMR spectra of 1 ml samples (7 mM phospholipid) were recorded on a Bruker MSL 300 spectrometer as described (de Planque, M. R., Kruijtzer, J. A., Liskamp, R. M., Marsh, D., Greathouse, D. V., Koeppe, R. E., 2nd, de Kruijff, B. & Killian, J. A. (1999) J. Biol. Chem. **274**, 20839-20846), acquiring 60,000 scans at 30°C.

**Results and Discussion**

**A novel method for the lipid encapsulation of cisplatin.**

The method of the present invention involves hydration of a dry lipid film composed of equimolar amounts of dioleoyl-phosphatidylserine (PS) and dioleoyl-phosphatidylcholine (PC), with a buffered solution (pH 7.4) of 5 mM cisplatin followed by one or more, preferably 10 freeze-thaw (FT) cycles, and removal of free (extravesicular) cisplatin by centrifugation.

The cisplatin-containing lipid suspension (cisPt-PS/PC) was extremely cytotoxic (**Fig. 1A**) with a typical $IC_{50}$, the drug concentration at which cell growth is inhibited by 50%, of ~2 nM as compared to 0.5 $\mu$M for the free drug (conventional cisplatin). A lipid suspension not loaded with cisplatin (blank) was not cytotoxic, and mixing conventional cisplatin with the blank lipid suspension did not increase the cytotoxicity of cisplatin.

**Lipid-coated nanoparticles of cisplatin**

Omitting the freeze-thaw step or leaving out the negatively charged PS in the lipid mixture, resulted in a dramatic decrease in the cytotoxicity of the

formulation (**Fig. 1B**). This dramatic decrease in cytotoxicity was paralleled by a similar decrease in the encapsulation efficiency suggesting a direct relation between drug-to-lipid ratio and cytotoxicity: omitting freeze-thawing or leaving out PS typically decreased the drug-to-lipid mole ratio 10-fold and 4-fold, respectively.

The cisplatin-to-lipid mole ratio was 0.5 ± 0.1 (n=17), which translates into a theoretical intravesicular concentration of cisplatin in excess of 30 mM (based on an encapsulated volume of 15 liter/mole phospholipid (cf. Hope et al. (1986), supra). This value by far exceeded the solubility limit of cisplatin (~8 mM in chloride-free media (cf. Riley, C. M. & Sternson, L. A. (1985) Analytical profiles of drug substances **14**, 78-105), and suggested that the lipid formulations contained aggregates of cisplatin. Indeed, cryo-EM examination (**Fig. 2A**) and elemental microanalysis (EDAX; data not shown) revealed the presence of small electron-dense particles containing platinum, and these nanoparticles of cisplatin were found to be coated by a thin lipid layer.

Much higher drug-to-lipid ratios of 2.5 ± 0.1 (n=4) were obtained when during the preparation of the cisplatin formulation, a solution of cisplatin in $H_2O$ instead of buffer was used. Under these conditions, nanoparticles were much more abundant and could be isolated using sucrose density centrifugation (**Fig. 2B**).

EM analysis of the gradient fractions showed that the pellet fraction existed of lipid-coated nanoparticles (**Fig. 2C**), and that lipid vesicles were virtually absent (data not shown). The majority of the particles was bean-shaped measuring 46 ± 16 nm by 86 ± 32 nm (lipid coat inclusive).

Negative stain EM showed electron-dense nanoparticles surrounded by a bright layer not accessible to the stain (**Fig. 2D**). This bright layer corresponds to the hydrated lipid coating. A single bilayer coat would be expected to have a thickness of ~6.6 nm, while a coat

consisting of two bilayers should be at least 10.4 nm thick (based on a bilayer thickness of 3.8 nm and a water layer of 2.8 nm; cf. Lewis, B. A. & Engelman, D. M. (1983) J. Mol. Biol. **166,** 211-217). Thus, the thickness of the layer, 5-9 nm for ~80% of the particles, indicated that most nanoparticles of cisplatin were coated by a single lipid bilayer.

The presence of a lipid coat was confirmed in $^{31}$P-NMR experiments (**Fig. 3A**) which showed a bilayer-type of spectrum typical for membranous particles of this shape and size (in accordance with Burnell, E. E., Cullis, P. R. & de Kruijff, B. (1980) Biochim. Biophys. Acta **603,** 63-69).

Disruption of the lipid coat leads to the immediate dissolution of the lipid-coated nanoparticles: resuspension of the pellet resulted in a colloidal solution with a milky appearance, which immediately turned transparent upon addition of detergent (**Fig. 3B**).

The drug-to-lipid mole ratio of the pellet fraction was exceptionally high, 11 ± 2 (n=18), which translates into ~3.3 mg cisplatin per μmol phospholipid.

The cytotoxicity of the pellet fraction was much greater than that of conventional cisplatin, and could be further enhanced by filtering the pellet fraction (**Fig. 4A**).

Extrusion of the pellet through polycarbonate filters resulted in a narrow size distribution with an average size of 127 nm (**Fig. 4B**). The cytotoxicity of the extruded pellet fractions was the highest measured so far, with a typical $IC_{50}$ of 0.3 nM i.e. about 1000-times more cytotoxic than conventional cisplatin tested in parallel (**Fig. 4A**).

**Table**

Factors determining cisplatin nanoparticle formation

| | Experimental Conditions | | | Nanoparticle Formation: |
|---|---|---|---|---|
| Lipid[¶] | Cisplatin | Medium ($H_2O$) | FT | Pellet[*] |
| PS/PC (1/1) Control | + | + | + | + (100 %) |
| None | + | + | + | - |
| PS/PC (1/1) | - | + | + | -[†] |
| PS/PC (1/1) | + | + | - | - |
| PC | + | + | + | - |
| PS/PC (1/1)[‡] | + | + | + | + |
| PA/PC (1/1) | + | + | + | + |
| PG/PC (1/1) | + | + | + | + |
| PS/PE (1/1) | + | + | + | + |
| PS/SM (1/1) | + | + | + | + |
| PS/SM/Chol (3/3/4) | + | + | + | + |
| PS/PC (1/1) | + | + 150 mM NaCl | + | - |
| PS/PC (1/1) | + | + NaOH (pH 8.0)[§] | + | - |
| PS/PC (1/1) | + | + 50 mM NaCl | + | +/- (15 %) |
| PS/PC (1/1) | + (2 % diaquo) | + 50 mM NaCl | + | +/- (50 %) |
| PS/PC (1/1) | + (10 % diaquo) | + 50 mM NaCl | + | + |
| PS/PC (1/1) | + | + 150 mM $NaNO_3$ | + | +/- (35 %) |

[*]Nanoparticle formation was determined by sucrose density centrifugation as the amount of cisplatin in the pellet fraction relative to that in the control pellet (PS/PC, 5 mM cisplatin in $H_2O$, FT): >70% (+), 15-50% (+/-), <10% (-). [†]Visual inspection. [‡]Cisplatin added to preformed liposomes (unilamellar or multilamellar). [§]5 mM cisplatin in $H_2O$ has pH 5.5 and contains ~10 % diaquated (aquo/hydroxo) species of cisplatin. [¶]Abbreviations: dioleoyl-phosphatidic acid (PA), -phosphatidylglycerol (PG), -phosphatidylethanolamine (PE), sphingomyelin (SM), cholesterol (Chol).

## CLAIMS

1. Method for encapsulating substances, in particular drugs, that have a low water-solubility and lipophilicity, which method comprises:

a) providing a lipid system comprising one or more negatively charged lipid(s);

b) combining the lipid system with the substance(s) in a medium at a low total solutes concentration;

c) subjecting the mixture thus obtained to one or more cycles of freezing and thawing to produce lipid-coated aggregates of the substance(s); and

d) optionally removing the free (non-enclosed) substance(s).

2. Method as claimed in claim 1, wherein the medium having a low total solutes concentration is a solution of the substance(s) having a maximum total solutes concentration of 0.1 molar.

3. Method as claimed in claim 1, wherein the medium having a low total solutes concentration is a solution of the substance(s) having a maximum total solutes concentration of 0.02 osmolar.

4. Method as claimed in claims 1-3, wherein the substance(s) is(are) used in a high concentration.

5. Method as claimed in claim 4, wherein the high concentration is a concentration that does not lead to the formation of macromolecular aggregates of the substance(s) in the solution of the substance(s).

6. Method as claimed in claim 4 or 5, wherein the high concentration lies close to the solubility limit of the substance(s).

7. Method as claimed in claims 1-6, wherein the lipid system is a single system consisting of one lipid.

8. Method as claimed in claim 1-6, wherein the lipid system is a mixed system comprising at least 20 mol%, preferably 50 mol% negatively charged lipid(s).

9. Method as claimed in claims 1-8, wherein combining the lipid system with the substance(s) is effected by hydrating a dry film of the lipid system with a solution of the substance(s).

10. Method as claimed in claims 1-8, wherein combining the lipid system with the substance(s) is effected by first hydrating a dry lipid film to obtain preformed liposomes and subsequently adding a solution of the substance(s).

11. Method as claimed in claims 1-10, wherein 5 to 10 cycles of freezing and thawing are performed.

12. Method as claimed in claims 1-11, further comprising the step of isolating the lipid-coated aggregates of the substance(s).

13. Method as claimed in claim 12, wherein the lipid-coated aggregates of the substance(s) are isolated by high-speed centrifugation (pelleting) through sucrose cushions.

14. Method as claimed in claim 12, wherein the lipid-coated aggregates of the substance(s) are isolated by low-speed centrifugation.

15. Method as claimed in claims 1-14, wherein the substance is the drug cisplatin.

16. Method as claimed in claims 1-14, wherein the substance is selected from magnetic resonance and X-ray imaging agents based on Europium of Gadolinium.

17. Method as claimed in claims 1-16, wherein the negatively charged lipids are selected from the group consisting of phosphatidylserine (PS), phosphatidic acid (PA), phosphatidylglycerol (PG), phosphatidylinositol (PI) and other negatively charged amphiphiles.

18. Method as claimed in claims 1-6 and 8-17, wherein the non-negatively charged lipids in a mixed lipid system are neutral lipids selected from the group consisting of phosphatidylcholine (PC), sphingomyelin (SM), cholesterol, phosphatidylethanolamine (PE), phosphatidylethanolamine derivatized with

polyethyleneglycol (PEG-PE) or other hydrophilic polymers, and other net neutral amphiphiles.

19. Method as claimed in claims 1-6 and 8-18, wherein the lipids in a mixed lipid system are phosphatidylserine (PS) and phosphatidylcholine (PC), carrying dioleoyl fatty acids.

20. Method as claimed in claims 1-19, wherein the solution containing the substance(s) to be encapsulated, in particular one or more drug(s), is a buffered solution of an approximately neutral pH.

21. Method as claimed in claim 20, wherein the pH of the buffered solution is about 7.4.

22. Method as claimed in claims 1-19, wherein the solution containing the substance(s) to be encapsulated, in particular one or more drugs, is water.

23. Method as claimed in claims 1-22, wherein the solution of the substance(s) further comprises one or more helper substance.

24. Method as claimed in claims 1-22, wherein the helper substance(s) is(are) positively charged.

25. Method as claimed in claims 1-24, wherein conditions are chosen such that positively charged species of the substance(s) (or helper substance(s)) are present in the solution of the substance(s).

26. Method as claimed in claims 1-25, wherein the freeze-thaw cycles are effected by freezing in ethanol/dry ice and thawing in a waterbath of 37°C.

27. Method as claimed in claims 1-26, wherein removal of the extravesicular drug is effected by repeated centrifugation and resuspension of the membrane pellet in a medium.

28. Method as claimed in claims 1-27, wherein the medium is compatible with human use.

29. Method as claimed in claim 28, wherein the medium is selected from the group consisting of water, aqueous solutions of synthetic buffers and aqueous solutions of buffers.

30. Method as claimed in claims 1-29, further comprising the step of sizing the lipid-coated aggregates of the substance(s).

31. Method as claimed in claim 30, wherein the step of sizing the lipid-coated aggregates of the substance(s) is effected by means of extrusion of a solution of the aggregates through a filter having a cut-off corresponding with the aggregate size to be obtained.

32. Method as claimed in claim 30, wherein the step of sizing the lipid-coated aggregates of the substance(s) is effected by means of sonication.

33. Method as claimed in claims 1-32 wherein instead of the lipid(s) one or more amphipathic compounds other than lipids are used.

34. Method as claimed in claims 1-33, wherein instead of the one or more cycles of freezing and thawing aggregate formation is induced by combining the lipid system with an oversaturated solution of the substance to be encapsulated and adding nucleation sites thereto.

35. Formulation of a substance that is poorly water-soluble and has a low lipophilicity, obtainable by the method as claimed in claims 1-34.

36. Formulation as claimed in claim 35, wherein the substance takes the form of aggregates thereof coated with one or more bilayer(s) of lipids.

37. Formulation as claimed in claim 36, wherein the majority of the aggregates are coated with one bilayer of lipids.

38. Formulation of cisplatin, wherein the cisplatin takes the form of aggregates of cisplatin coated with one or more bilayer(s) of lipids.

39. Formulation of cisplatin as claimed in claim 38, wherein the majority of the aggregates are coated with one bilayer of lipids.

40. Formulation of cisplatin as claimed in claims 38 and 39, wherein the aggregates of cisplatin are of an elongated shape measuring about 46 nm by 86 nm (lipid coat inclusive).

41. Formulation of cisplatin as claimed in claims 38-40, obtainable by the method as claimed in claims 1-34.

42. Formulation of cisplatin obtainable by the method as claimed in claims 1-34.

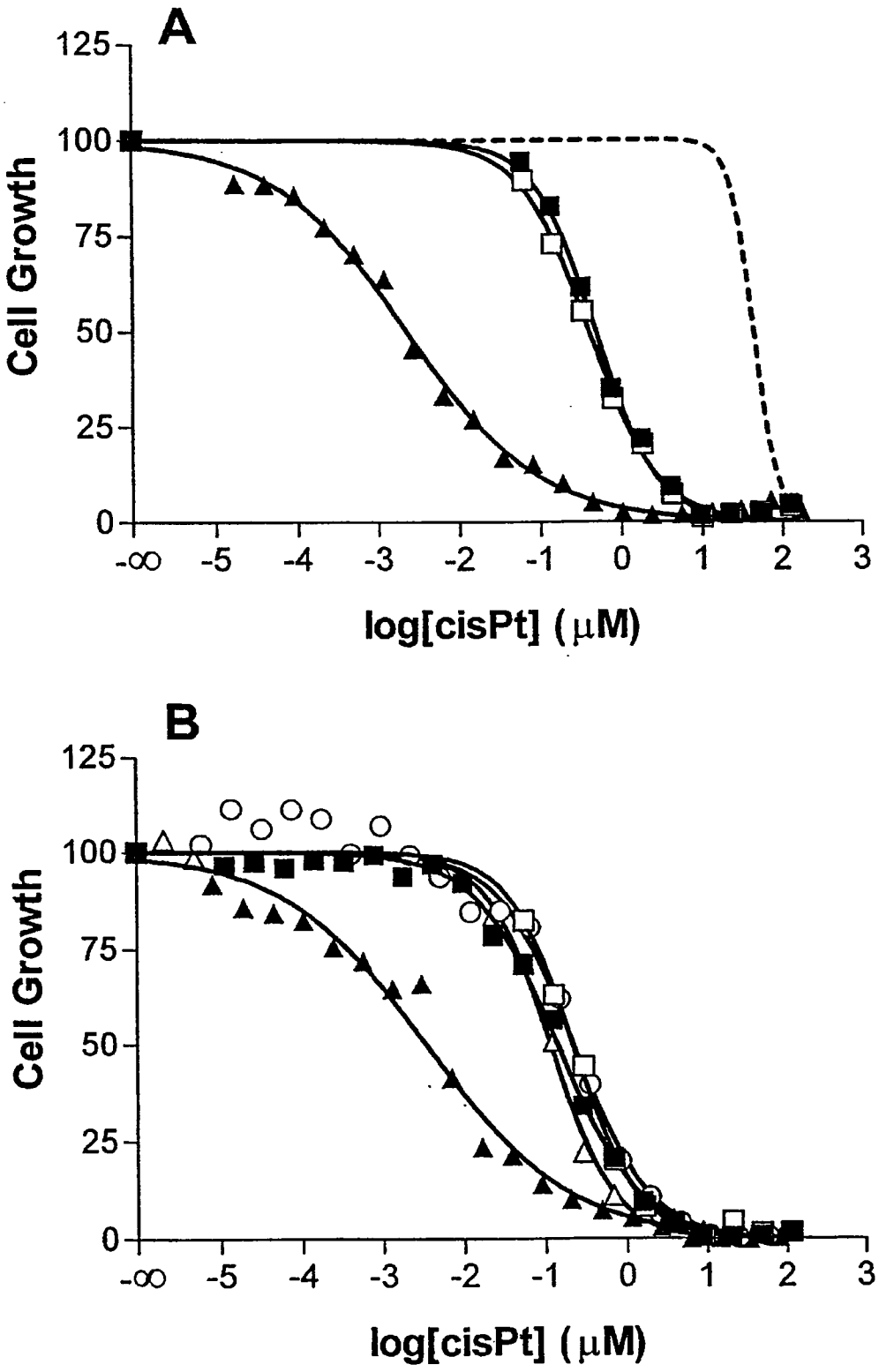

A
125
100
75
50
25
0
Cell Growth
-∞ -5 -4 -3 -2 -1 0 1 2 3
log[cisPt] (μM)
B
125
100
75
50
25
0
Cell Growth
-∞ -5 -4 -3 -2 -1 0 1 2 3
log[cisPt] (μM)

Fig. 1

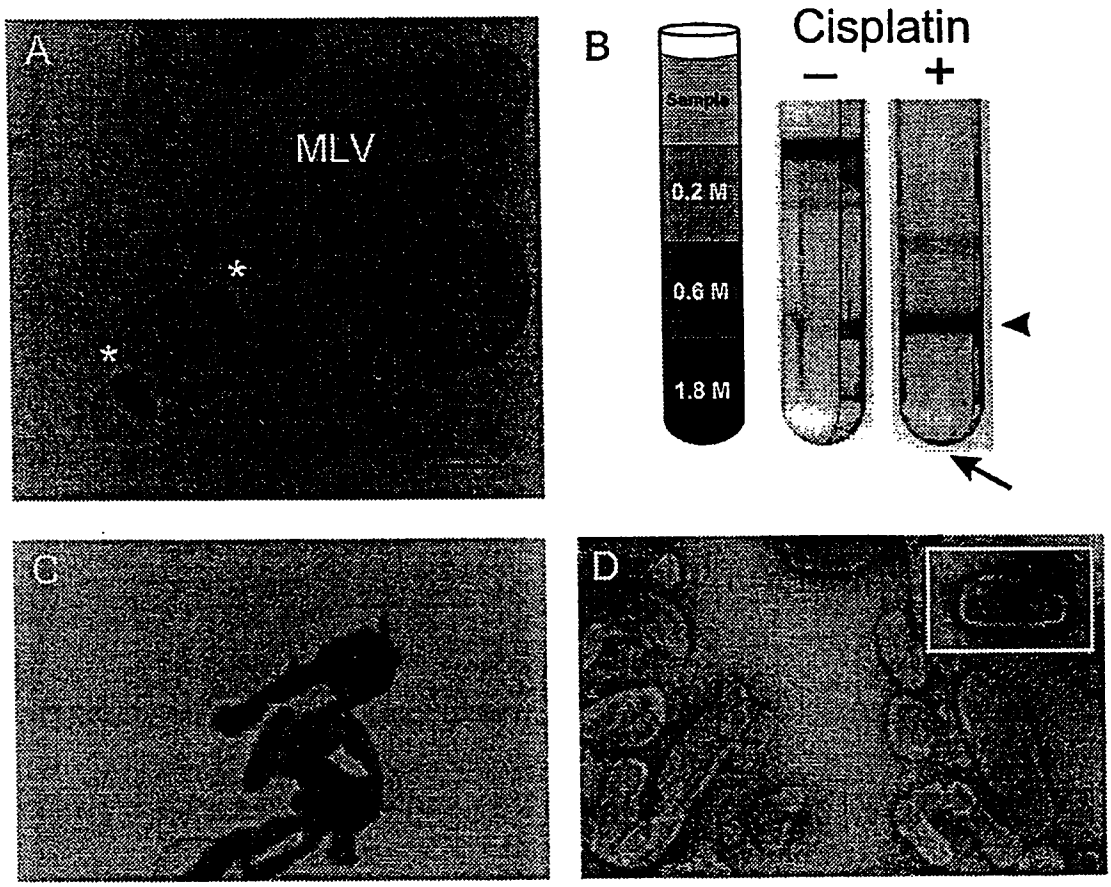
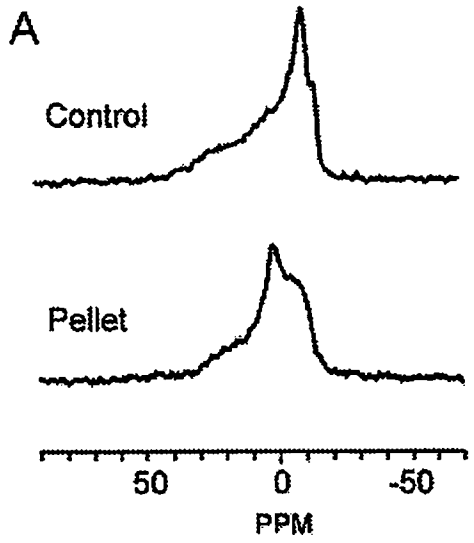



Fig. 2



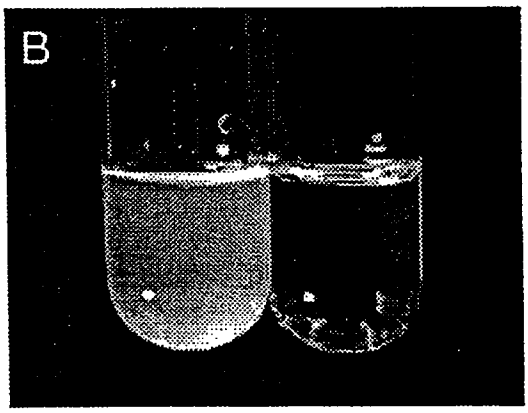




Fig. 3

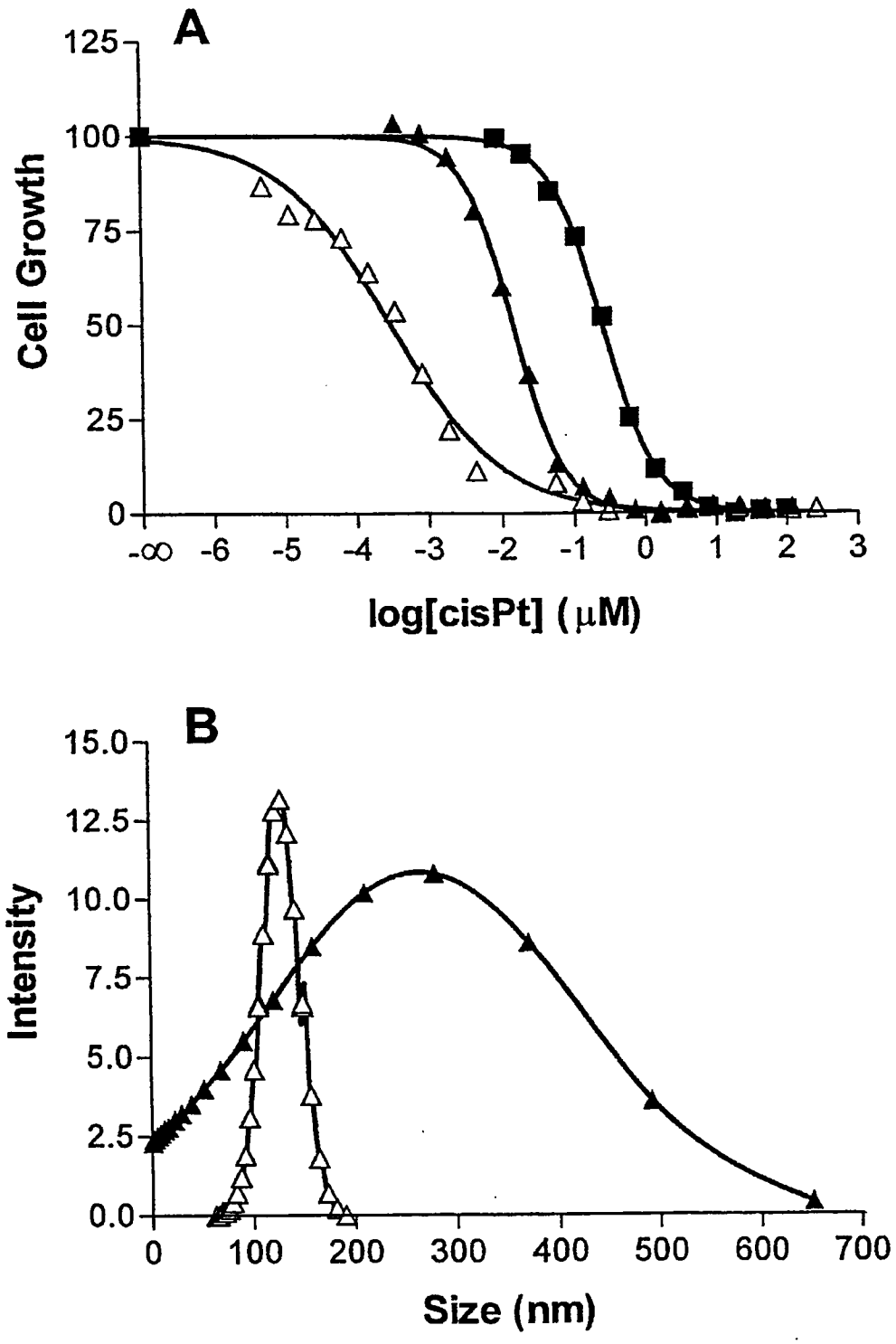

A
125
100
75
50
25
0
Cell Growth
-∞ -6 -5 -4 -3 -2 -1 0 1 2 3
log[cisPt] (μM)
B
15.0
12.5
10.0
7.5
5.0
2.5
0.0
Intensity
0 100 200 300 400 500 600 700
Size (nm)

Fig. 4

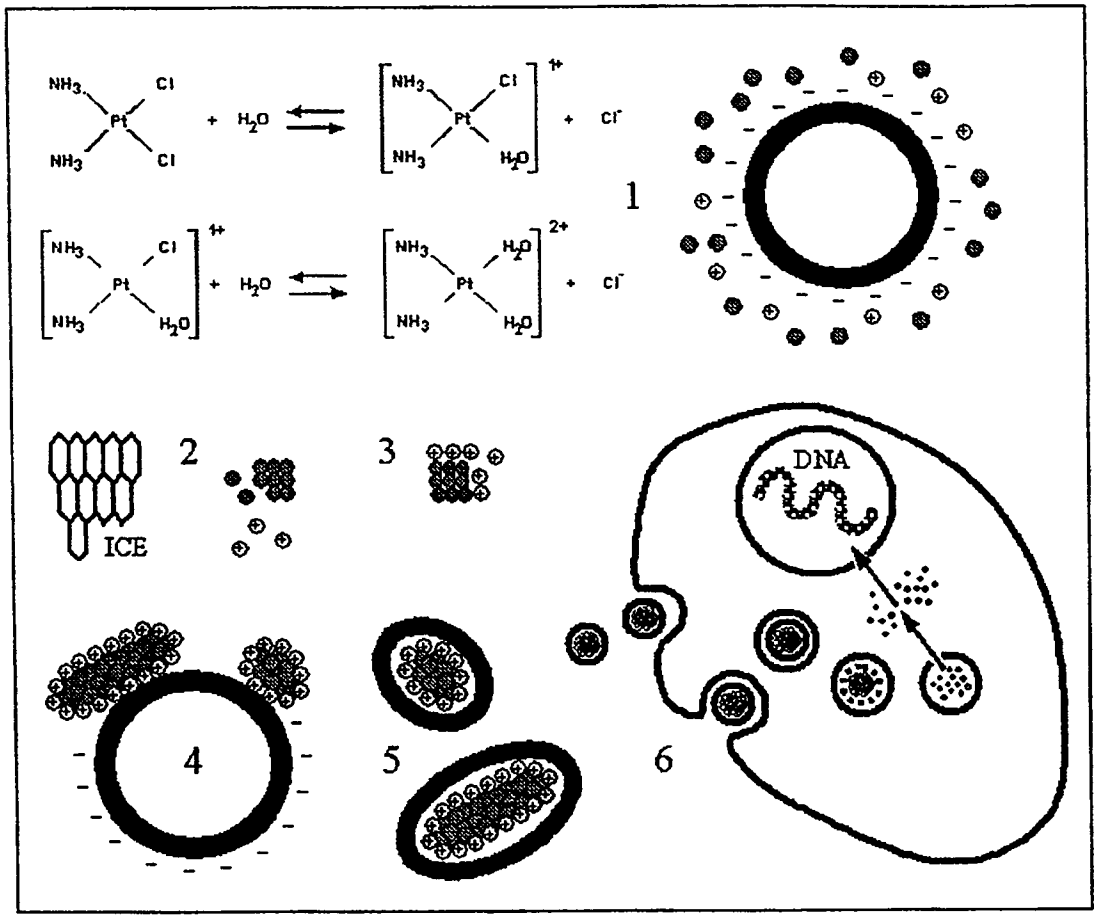



Fig. 5